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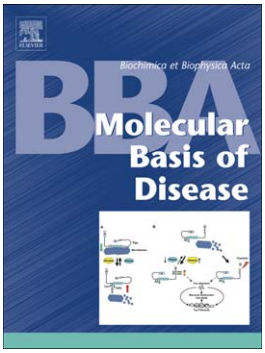

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LIVER X RECEPTORS, LIPIDS AND THEIR REPRODUCTIVE SECRETS IN THE MALE

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Abstract

Liver X receptor (LXR) α and LXRβ belong to the nuclear receptor superfamily. For many years they have been called orphan receptors, as no natural ligand was identified. In the last decade the LXR natural ligands have been shown to be oxysterols, molecules derived from cholesterol. While these nuclear receptors have been abundantly studied for their roles in the regulation of lipid metabolism, it appears that they also present crucial activities in reproductive organs such as testis and epididymis, as well as prostate. Phenotypic analyses of mice lacking LXRs (*lxr*−/−) pointed out their physiological activies in the various cells and organs regulating reproductive functions. This review summarizes the impact of LXR-deficiency in male reproduction, highlighting the novel information coming from the phenotypic analyses of *lxrα*−/−, *lxrβ*−/− and *lxrα;β*−/− mice.

Key-words: Testis, epididymis, prostate, LXR, lipids

1. LXRs AT A GLANCE…

In the early 90's, the discovery of numerous nuclear receptors, called "orphan" since no *bona fide* ligand had been identified, opened the way of the reverse endocrinology [1]. In contrast to classical endocrinology where the effector is discovered following the study of its hormone, the nuclear receptor is used to screen for ligands, either natural or not, which modulate its transcriptional activity. The ligand, in turn, is used as a chemical tool to dissect the role of its nuclear receptor in physiology and pathophysiology [2]. Over the past decade, reverse endocrinology has been used to link several orphan receptors to ligands and biological functions. Such philosophy has led to the identification of liver X receptors (LXRs) α ([3]; NR1H3) and β ([4, 5]; NR1H2) as oxysterol receptors [6, 7], and to deciphering of their physiological functions. In turn, synthesis of non-metabolisable molecules modulating their transcriptional activity, permitted the investigations of their putative interest as pharmacological targets [8].

LXRα and LXRβ form obligatory heterodimers with retinoid receptors (RXR, NR2B1-3), the receptors of 9-*cis* retinoic acid [3, 9]. LXRβ was found to be expressed in many tissues, whereas LXRα is expressed mainly in a restricted subset of tissues known to play an important role in lipid metabolism (such as liver, small intestine, kidney, spleen, and adipose tissue; for a review see [10]). In absence of ligand, LXRs constitutively bind to RXRs and specific binding sequences localized on target gene promoters [3], together with co-repressors, which block transcription by recruitment of histone deacetylase. Hence, the presence of the complex [RXR/LXR-corepressor-histone deacetylase] on the DNA usually acts as a basal repressor of gene transcription [9]. Oxysterol or 9-*cis* retinoic acid binding to their respective nuclear receptors leads to modifications of the ligand binding pocket within the carboxy-terminus domain. This induces the release of the co-repressors and reinforces the interactions with the co-activators [11]. This cascade of events allows the recruitment of proteins with acetyl-transferase activity and a permissive chromatin environment, which finally enhances LXR-target gene expression and thus the physiological response of the cell.

A large number of natural LXR ligands have been described, such as oxidized derivatives of cholesterol (for a review see [12, 13]). In mammals, the main source of oxysterols remains

endogenous production [13]. An important enzyme of this pathway is the sterol 14α-demethylase (EC 1.14.13.70, CYP51), a cytochrome P450 required for sterol biosynthesis in different phyla, and the most widely distributed P450 gene family being found in all biological kingdoms [14]. It catalyzes the first step following cyclization in sterol biosynthesis such as removal of the 14 alpha-methyl group from lanosterol in the cholesterol biosynthetic pathway (Figure 1). Interestingly, although the human 14α-demethylase gene is expressed in a variety of tissues, the highest levels are observed in testis, ovary, adrenal, prostate, liver, kidney, and lung. In the reproductive tract, many activating oxysterols are present (for a review see [13]): including 22(R)-hydroxycholesterol (within the steroidogenic pathway), follicular fluid meiosis activating sterol (FF-MAS), and its derivative, testis meiosis-activating sterol (T-MAS).

Due to the lipid nature of the ligands, the physiological roles of LXRs have been extensively detailed in the homeostasis of cholesterol in the gut-liver axis [15]. The role of the LXRs on cholesterol metabolism was determined using engineered knock-out mice lacking one (*lxrα*−/− or *lxrβ*−/−) or both (*lxrα;β*−/−) isoforms. Historically, the first analyses were performed on the *lxrα*−/− mice, which developed a hepatic steatosis, due to cholesteryl ester accumulation when fed a cholesterol-rich diet [16]. The molecular mechanism leading to this phenotype was the lack of up-regulation of *cyp7a1* encoding for the rate-limiting enzyme for the metabolism of cholesterol into bile acid (Figure 1). *Lxrβ*−/− [17] and *lxrα;β*−/− [17, 18] mice were then obtained. The role of the LXRs in cholesterol metabolism was thus extended to *de novo* synthesis of cholesterol [19], excretion [16] and detoxification of bile acids [20] or lipids [21], as well as in glucose homeostasis [22], immunity [23], skin development and homeostasis [24] and brain functions [25, 26]. LXRs, by regulating expression of several genes (including *ABCA1* [18, 27], *ABCG1* [28], apolipoprotein E (*APOE*) [29], and *PLTP* [30]) also play a critical role in reverse cholesterol transport. Interestingly, activation of LXRs in intestine and macrophages efficiently prevents atherosclerosis [31, 32]. This review will emphasize the physiological roles of LXRs in the male and thus focus on testis, epididymis and prostate (Figure 2).

The hypothesis that LXRs could also have physiological roles in steroidogenic and reproductive tissues came from difficulties in maintainance of the mouse colony, as well as

from previous studies performed on the adrenals [33, 34]. LXRα-deficient mice presented an adrenomegaly due to a higher cholesteryl ester content and a Cushing-like syndrome, as shown by the increased levels of blood corticosterone [33]. This work emphasized the role of LXRα as an important regulator of adrenal cholesterol homeostasis through its ability to modulate transcription of genes that govern the three major pathways of adrenal cholesterol, namely efflux, storage, and conversion into steroid hormones [33]. *In vivo* studies also showed that LXRα- and LXRβ-deficient mice had reduced fertility, characterized by less frequent conception and lower number of pups per litter [35, 36]. Careful examination confirmed that both sexes were affected by reproductive abnormalities. Female mice showed i) ovarian hyperstimulation-like syndrome [37], a syndrome characterized in women by ovarian enlargement associated with an extra-vascular fluid concentration, haemorrhagic ovarian *corpora lutea* and elevated estradiol serum levels [38, 39], as well as ii) parturition defects due to abnormal uterine contraction [40]. LXR-deficient males present abnormal features both within the testis and epididymis (see following chapter). Besides, experiments performed on human cell culture suggested that LXRs could have a protective effect in prostate cancer (see chapter 4.1).

2. LXRα AND LXRβ ARE INVOLVED IN VARIOUS PHYSIOLOGICAL PROCESSES IN THE TESTIS

Investigation of LXR-double knock-out mice revealed a decreased fertility at 5 months of age and evolving to complete infertility by 9 months [35, 36]. Several testicular functions have been found impaired in LXRα- and LXRβ-deficient mice: (1) steroidogenesis, (2) lipid metabolism and (3) proliferation/apoptosis balance in germ cells (Figure 2).

Quantitative PCR analysis of both LXR isoforms showed that LXRα is expressed in Leydig cells, while LXRβ was found in Sertoli cells, suggesting a specific role of each isoform. Both LXRs are present in the germ cells.

2.1. LXRα is involved in germ cell apoptosis while LXRβ controls their proliferation

Spermatogenesis is maintained by a delicate balance between proliferation, differentiation, and death of germ cells. Alteration of these processes results in spermatogenic impairment and thus infertility. Both proliferation and apoptosis were found altered in LXR-deficient mice [36].

Analysis of the single LXR-KO mice showed that LXRα is involved in the regulation of apoptosis in the testis [36]. TUNEL analyses revealed that *lxrα*-/- (as well as *lxrα;β*-/-) mice had a significantly higher number of apoptotic cells compared with wild-type mice, whereas a slightly but not significantly decreased number of apoptotic cells was observed in *lxrβ*-/- mice. Consistent with these data, mRNA expression analyses showed a higher accumulation of the proapoptotic transcript Bad, as well as TNFα in LXRα-lacking mice. Conversely, LXRβ-deficient (as well as *lxrα;β*-/-) mice had a significantly lower number of proliferating cells [36] and *cyclinA1* mRNA accumulation, suggesting that LXRβ is involved in germ cell proliferation. Infertility and destructured testis were observed only when both isoforms were absent [36]. A schematic view of a testis tubule with the various cells and the proteins, whose accumulation was altered, is given in figure 3.

2.2. LXRα controls androgen synthesis in testis

The hypothesis that LXRα could regulate androgen production came from the decreased level of testicular testosterone observed in *lxrα*-/- and *lxrα;β*-/- mice [36]. Type 1 3β-hydroxysteroid dehydrogenase isomerase (*3βhsdI*) mRNA accumulation was the most affected of the mRNA encoding the steroidogenic proteins (Figure 3), whereas levels of steroidogenic acute regulatory protein (*StAR*) and the cytochromes 11a1 (*cyp11a1*) and 17 (*cyp17*) transcripts remained unchanged (refer to figure 1 for the proteins). Moreover, significantly lower plasma concentrations of luteinizing hormone (LH) were found in LXRα-deficient mice [36]. These data were confirmed by lower level of expression of the specific β-chain of LH in the pituitary of these animals. Additionally, LXRα-deficient mice were able to respond to human chorionic gonadotropin challenge by an increased production of testosterone

similar to their wild-type controls. Interestingly, LXR agonist T0901317 increased testosterone concentration in wild-type mice (almost 14-fold compared to the vehicle-gavaged mice), as well as accumulation of StAR at both mRNA and protein levels. Together, these data indicate that LXRα regulates steroid synthesis not only in adrenal cells [33] but also in Leydig cells [36].

2.3. Both LXRα and LXRβ play a crucial role in lipid homeostasis in the testis

Part of the phenotype observed in the LXR-deficient mice was correlated with an alteration of lipid homeostasis [35, 36, 41]. The main enzymes involved in the fatty acid pathway are indicated on Figure 4. mRNA levels of sterol response element binding protein-1c (*srebp1c*) and fatty acid synthase (*fas,* Figure 4), encoding the sterol response element binding protein-1c and the fatty acid synthase, respectively, were decreased by 40% in *lxrα;β-/-* mice (Figure 3) compared to the wild-type mice [36]. In contrast, the level of *scd1*, encoding the stearoyl CoA-desaturase 1 (Figure 4), was increased by 2-fold in LXR-deficient mice (Figure 3), while *srb1*, encoding the scavenger receptor B1, *abca1* (ATP-binding cassette, sub-family A member 1), and *scd2* (Figure 4) were unchanged [36]. Interestingly, oil-red O staining pointed an accumulation of lipids in the Sertoli cells and in spermatids of LXR-deficient mice. These observations confirmed that fatty acid metabolism is important for reproductive functions, as previously reported [42]. It could also be concluded that lipid homeostasis alteration was the first event in this long process of testis disorganization in *lxrα;β-/-* mice [36], as suggested by Mascrez et al. [35].

Our data also showed that the lack of both LXRα and LXRβ leads to an increase of RARα and RARβ (all-*trans* retinoic acid receptors, NR1B1 and NR1B3), and retinaldehyde dehydrogenase-2 (RALDH-2) expressions [36] (Figure 3), resulting in deregulation of retinoic acid signaling. This is seen in the expression pattern of known RAR-target genes, such as dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (*dmc1*) and synaptonemal complex protein 3 (*scp3*), and could lead to spermatogenic disorders. Lipid accumulation has previously been observed in rat Sertoli cells in hypervitaminosis A [43], suggesting links between retinoid and lipid pathways. How the lack of LXRs act upon the retinoic acid signaling pathway remains to be clarified; however it could be

hypothesized that SHP (small heterodimeric partner, NR0B2), a non-canonical orphan nuclear receptor, could play a major role as shown by Volle *et al*. [44, 45] by studying SHP-deficient mice. Indeed, SHP has been described to be a negative regulator of a number of nuclear receptors such as LXRs and RARs [46].

Phenotypic analysis of *lxr-/-* mice has thus shown that cooperation between LXRα and LXRβ maintains both testis structure and function. In human, Chen *et al*. [47] identified and characterized two alternative spliced transcript variants of LXRα. LXRα2, which has a shorter N-terminal domain a reduced transcriptional activity, was found highly expressed in testis. The physiological role of the shorter form remains to be defined in man. Since ablation of LXRs impairs the fertility of aging mice, a putative defect in LXR-signaling cannot be excluded in the premature loss of fertility observed in some men.

3. LXR-DEFICIENT MICE PRESENT ABNORMAL FEATURES OF THE EPIDIDYMIS

As described above, *lxrα;β−/−* male mice become completely infertile when the animals reach the age of 9 months. The infertility arises from the association of testicular alterations [36] with an epididymal destructuration [48] observed in the two first segments of the organ (for a schematic representation see figure 5), which functions in regulation of the cholesterol homeostasis and maturation of spermatozoa. The phenotype observed in the *lxrα;β−/−* mice is characterized by an enlargement of the tubule lumen, with the presence of an amorphous substance in the lumen and shrinkage of the epithelial height. Interestingly a 15-day supplementation with androgens could not reverse the phenotype. Oil-red-O staining of *caput* epididymidis cryosections reveals lipid accumulation in the peritubular and interstitial tissues and the epithelium of *lxrα;β-/-* male mice. The amorphous substance in the tubule lumen was not stained, thus indicating that it was not composed of neutral lipids. Many isolated spermatozoa heads and flagella were observed when sperm were retrieved from the *cauda* epididymidis, revealing that the gametes were fragile, probably as a result of both testicular and epididymal dysfunctions [48]. The expression levels of genes regulating the fatty acid metabolism also seemed to be affected since quantitative real time RT-PCR showed that *srebp-1c*, *scd-1* and *scd-2*

mRNAs were decreased in *lxrα;β-/-* male caput epididymidis compared to wild-type mice. However, the impact of these down regulations was moderate as they do not influence fatty acid compositions of separated phospholipid and neutral lipid fractions in *lxrα;β-/-* animals [49]. Further investigations revealed that cholesterol trafficking was a LXR-regulated mechanism in mouse *caput* epididymidis, in a segment- and cell-specific manner [50]. In LXRα- and LXRβ-deficient animals, apical cells present in the two first *caput* segments had their cytoplasm filled with cholesteryl-ester droplets, in association with a loss of ABCA1 in the apical membrane of the apical cells (figure 5). The level of apoptotic apical cells was also increased in *lxrα;β-/-* compared to wild-type mice. ABCA1 was thus confirmed to be an important factor in the male reproductive tract, as male mice invalidated for this gene were previously shown to have a 21% fertility decrease over their lifespan. Both expression and location of ABCG1 were different from ABCA1 and were not altered in the epididymis of LXR-deficient mice. ABCG1 was present at the apical pole of all epithelial cells in the proximal caput epididymal segments [50], suggesting complementary functions for these two cholesterol transporters in the epididymal epithelium. These locations raise the question how cholesterol efflux could be involved in sperm maturation.

Even though germ cells already presented abnormal lipid accumulation in the testis [36], alterations of cholesterol homeostasis may also be linked to sperm maturation defects along the epididymal duct and lead to impaired fertility. In man, dyslipidemia, obesity and/or hypercholesterolemia are generally associated with testicular defects and endocrine perturbations whereas defaults in epididymal sperm maturation are rarely investigated in these situations. Recent data showed that fertile three month-old *lxrα;β-/-* male mice became infertile when fed a 1.25% cholesterol containing diet during four weeks. An atherosclerosis-like process was observed in the proximal epididymis, provoking sperm morphological abnormalities, decreased motility and viability and premature acrosome reaction (Ouvrier *et al.* submitted). This study brings forward the epididymis as an early target of cholesterol toxicity in a dyslipidemic mouse model, and shows that post-testicular sperm alterations may be associated with male infertility under dyslipidemic conditions.

4. PHYSIOLOGICAL ROLE OF LXRα AND LXRβ IN PROSTATE CANCER

Prostate cancer is the most frequently diagnosed cancer and the leading cause of death from cancer in men over 50 years old. Among the various genetic and environmental risk factors, epidemiological analyses have revealed a positive association between hypercholesterolemia and the development of prostate cancer [51, 52]. Indeed, epidemiological studies have shown that Chinese populations, with a low risk to develop prostate cancer, had an increased risk after migration to the United States. This environmental effect was attributed to the deleterious impact of lipid consumption on this cancer [53]. Actually, cholesterol accumulation in tumors was first reported in the early 20th century [54] without any clear mechanistic explanation [55]. One of the various hypotheses was that rapidly proliferating cancer cells require new components to build *de novo* plasma membrane. Consistent with this hypothesis HMG-CoA reductase inhibitors that impede *de novo* synthesis of cholesterol block prostate cancer cell growth *in vitro* [56]. Statins and their derivatives have thus been suspected to have benefits in prostate cancer progression in patients undergoing long-term treatment [57-59]. Eventhough LXRs are key-sensors of cholesterol homeostasis, their role in prostate physiology remains poorly understood.

4.1. Both LXRα and LXRβ modify the apoptosis/proliferation balance in prostate cancer cells.

Fukuchi *et al*. [60] first reported the control of proliferation by LXRs on LNCaP human prostate carcinoma cell line, *in vitro* as well as *in vivo*. In their experiments, LXR agonist T0901317 decreased the percentage of cells in S-phase through an up regulation of $p27^{kip1}$. The induction of expression of the cholesterol membrane transporter ABCA1 by T0901317 led to the assumption that ABCA1 was the key-regulator of the cell cycle in response to LXR activation [61]. Freeman and Solomon proposed that a critical cholesterol concentration in the membrane was required to allow raft coalescence [62]. Sequestration of “oncogenic” signaling proteins in a restricted area through raft coalescence could enhance their activity by exclusion of negative regulators outside the rafts [63, 64]. Based on that hypothesis, we explored whether LXRs could modulate cholesterol concentration in rafts [65]. *In vitro* and *in vivo* analyses revealed that modulation of LXR activity triggered apoptosis of prostate cancer

cells. This effect involves both the increase of cholesterol efflux by ABC proteins and the disruption of lipid-rafts signaling activity. Schematically (Figure 6), LXRs first mediate upregulation of ABCG1 that stimulates reverse cholesterol transport. This results in a reduction in plasma membrane cholesterol steady state levels. Then, both disruption of lipid-rafts and down-regulation of raft-associated signaling in prostate cancer cells are induced, together with a decrease in the phosphorylated fraction of raft-associated AKT. Cholesterol replenishment prevents entry of the cells into apoptosis in the presence of T0901317 demonstrating that cholesterol homeostasis regulation by LXRs is a key-process to control cell death. Consistent with this mechanism, chronic T0901317 treatment down regulates AKT and stimulates apoptosis of LNCaP derived tumors in xenografted mice [65]. These results pointed out that LXRα and LXRβ are important modulators of prostate cancer cell survival. Altogether, these findings reinforce the idea to consider LXR agonists as potential pharmacological agents in cancer prevention and anti-cancer therapy (for a review see [66]). Various studies indeed enlighten the anti-proliferative and pro-apoptotic effects of LXR-ligand on ovarian [67] and breast [68, 69] cancer models.

4.2. LXRα and benign prostatic hyperplasia

Benign prostatic hyperplasia concerns 50% of men over the age of 50 years [70]. Symptoms include urinary frequency, urgency incontinence (compelling need to void that cannot be deferred), and voiding at night (nocturia) [71]. Kim *et al*. [72] showed that LXRα-/- mice presented benign prostatic hyperplasia-like features on ventral prostate such as proliferative epithelial cells, multiple layers of dense stroma around the prostatic ducts and dilated prostatic ducts. These data suggest that LXRα agonists could also be useful in the treatment of this potentially harmful pathology since some patients may eventually progress to renal failure.

5. ARE LXR PROMISING PHARMACOLOGICAL TARGETS IN HUMAN DISEASES?

The discovery of new regulated transcription factors has always opened several fields of investigation. From an academic point of view, it is elegant to identify novel *bona fide* genes and associate the discovered factor to new physiological functions. The use of transgenic animals (fly, mouse, worm…) usually helps in linking abnormal features of the transcription factor (mutation or abnormal signaling pathway) to human diseases. At last, once this milestone is reached, chemists can synthesize thousands of new ligands in order to modulate the protein activity. However, the main concern for pharmacology researchers is to solve the pathological problem without opening Pandora's box to optimize the ligands of therapeutical interest without inducing major side effects (for a review see [66]).

Studies on mice pointed out that LXR-deficiency could be associated with several phenotypes resembling putative diseases found in human such as metabolic disorders, reproductive failures, central nervous system alterations, or various types of cancer [66]. Clinical use of LXR agonists should thus theoretically be useful in reducing cholesterol levels, neural degeneration, parturition defects, cancer progression… Up to now the major side effect of LXRs is a hypertriglyceridemia due to their activity in the liver on the fatty acid synthase. In analogy with what was done for the estrogen receptors, it is likely that SLiMs (Selective Liver X Receptor Modulators) need to be developed [66]. They should have tremendous therapeutical possibilities, after having successfully undergone the extensive approval process. In a near future…

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Figure legends

Figure 1. Schematic representation of cholesterol synthesis and metabolism into androgens or bile acids. 3-hydroxy-3-methyl-glutaryl-CoA (HMGCoA) reductase is the rate-controlling enzyme of the mevalonate pathway that produces cholesterol and other isoprenoids. This synthesis could virtually occur in all cells. Androgens synthesis mainly takes place in Leydig cells, bile acids synthesis in hepatocytes. Structures of the main sterols are indicated: lanosterol, cholesterol, T-MAS, 22(R)-OH-cholesterol and 7α-OH-cholesterol. For more details about the indicated enzymes see text. StAR, steroidogenic acute regulatory protein; CYP51, 14α-demethylase; CYP7A1, cytochrome P450 cholesterol 7α-hydroxylase; CYP11A1, cytochrome P450 side chain cleavage; CYP17, cytochrome P450 17α-hydroxylase/17,20-lyase; 3βHSD, 3β-hydroxysteroid dehydrogenase type 2.

Figure 2. Physiological roles of LXRs in male genital tract. Three main organs are targeted by LXR-disruption in male mice: testis, epididymis, and prostate. Schematically, LXRs regulate lipid homeostasis in testis and epididymis, as well as apoptosis-proliferation equilibrium of spermatozoa (spz), testicular germ cells and prostate epithelium. For more details see text.

Figure 3. Proteins which accumulation is altered by LXR-deficiency in the testis tubule.

Schematically, LXRs regulate lipid homeostasis in testis, as well as apoptosis-proliferation equilibrium of spermatozoa (spz) and testicular germ cells. For clarity, fibroblasts and myoid cells below the basal lamina have been omitted. Likewise, cytoplasmic bridges between secondary spermatocytes and between early spermatids are not shown. Decreased accumulation is indicated in red; increased accumulation in indicated in green; ABC, ATP-binding cassette protein; Bad, Bcl-2 associated death promoter protein; StAR, steroidogenic acute regulatory protein; RAR, all-*trans* retinoic acid receptor; RALDH, retinaldehyde dehydrogenase; SCD1, stearoyl Coenzyme A desaturase 1; SREBP, sterol regulatory element binding protein; TNF, tumor necrosis factor. Adapted from [73].

Figure 4. Schematic representation of fatty acid synthesis in mammals. Acetyl-CoA carboxylase (ACC) is the rate-limiting (committed) step in fatty acid synthesis. There are two major isoforms of ACC in mammalian tissues; FAS, fatty acid synthase ; SCD1/2, Stearoyl CoA desaturase 1 or 2; ELOVL, elongation of very long chain.

Figure 5. Schematic representation of the role of LXRs in the *caput* epididymidis. Epididymis is organized in three parts (*caput*, *corpus* and *cauda*). The main cells are indicated. The major phenotype of the LXR-deficient mice is observed in the segments I and II of the *caput* with the lack of ABCA1 (indicated in red) in the apical membrane of the apical cells. Latin numerals indicate the *caput* segments.

Figure 6. Role of LXRs in apoptosis of prostate human cancer cells.

A). When the level of cholesterol is high, it accumulates in membranes within the lipid rafts, which allows the growth factors to access to their receptors. Binding of these peptides increases cell proliferation and inhibits cell death by apoptosis. B) Activation of LXRs by its ligand induces a higher production of ATP-binding cassettes (ABC) involved in cholesterol efflux, which destructures the lipid rafts. Growth factors are less efficient to maintain cell proliferation, which in turn increases apoptosis. Broken arrow indicates an inhibition.

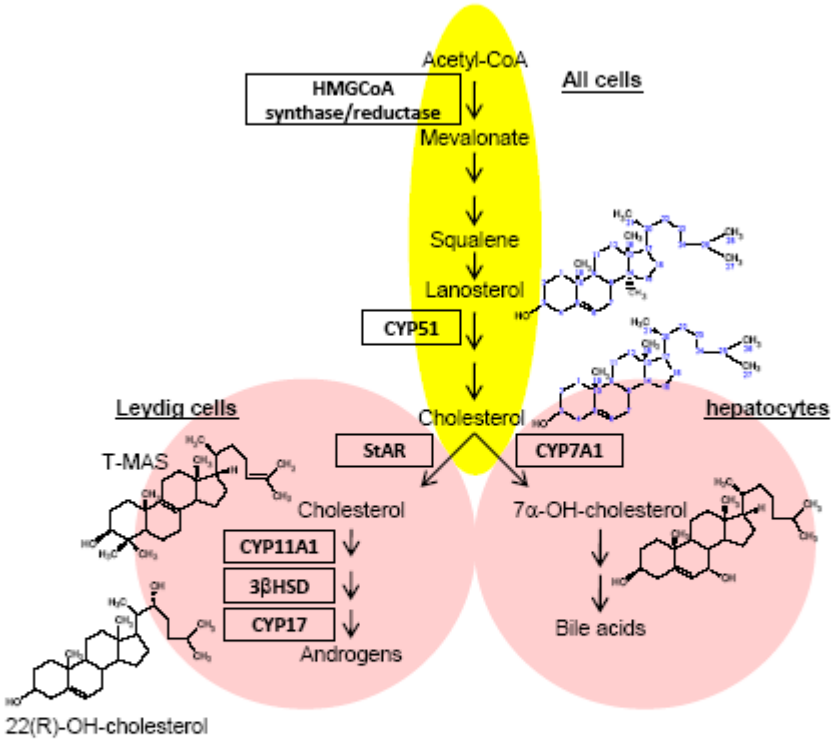

Figure 1.
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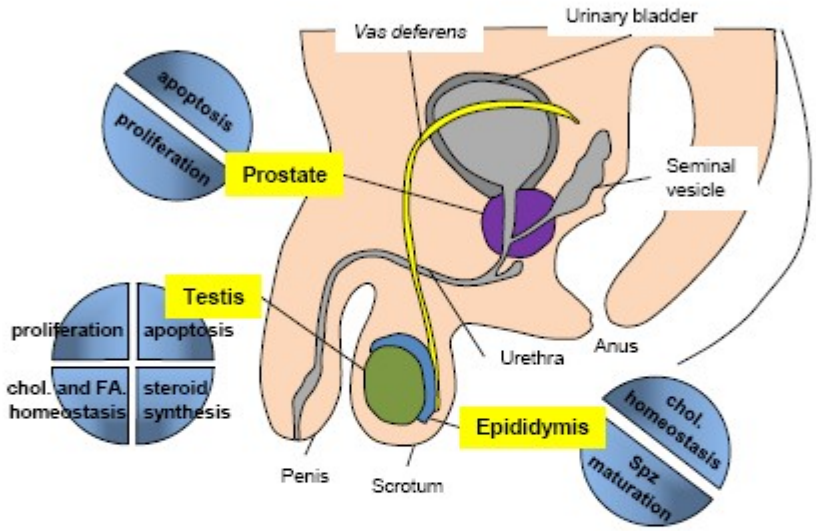

Figure 2.
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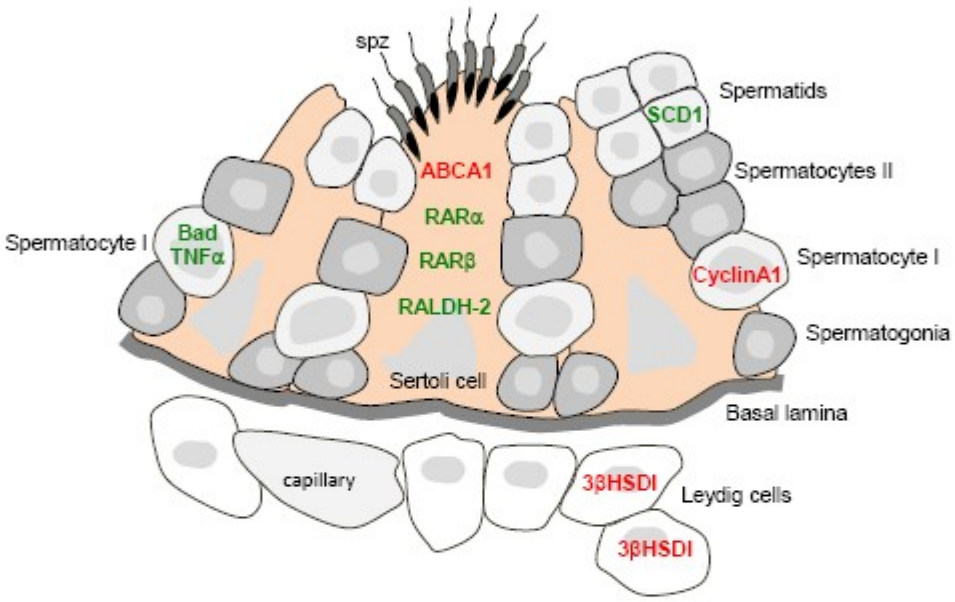

Figure 3.
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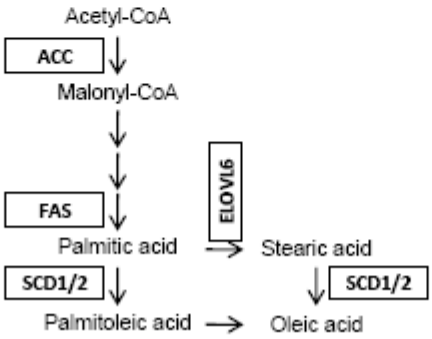

Figure 4.
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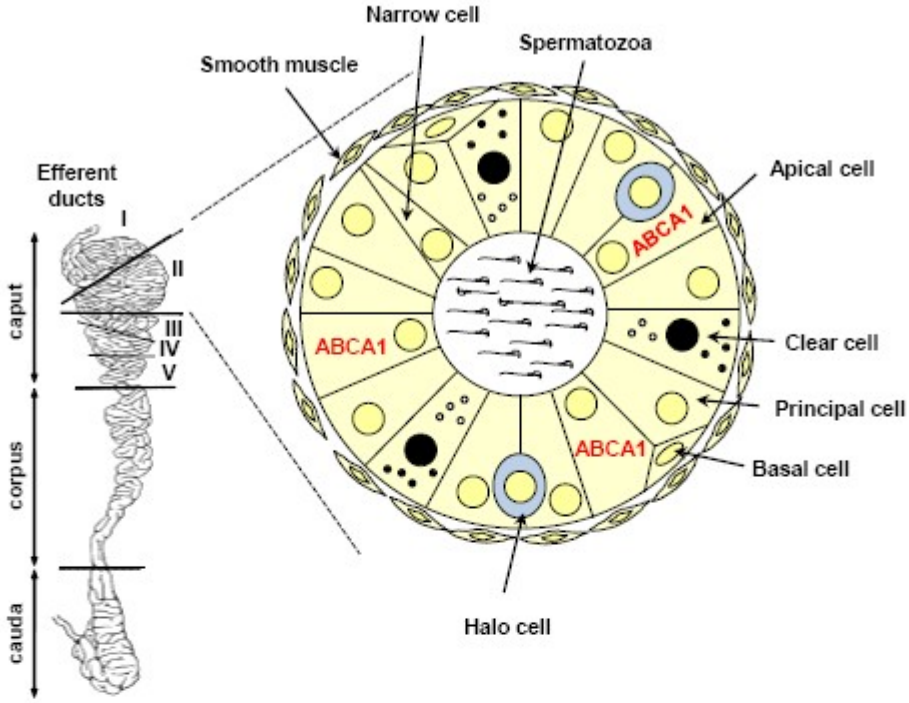

Figure 5.
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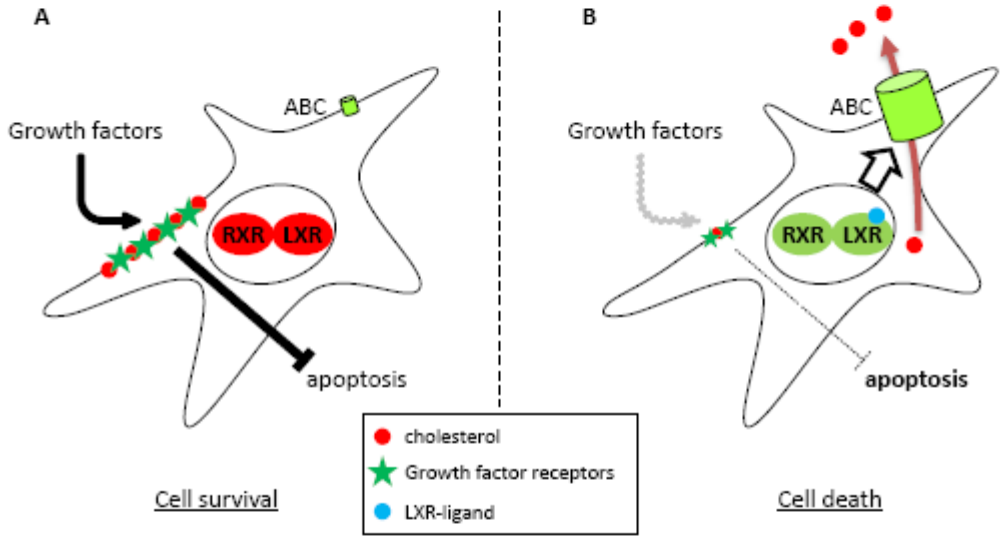

Figure 6.
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